EFFECTS OF STIFFNESS GRADIENT AND HYDROSTATIC PRESSURE ON CELL MIGRATORY BEHAVIORS

BY

DANIELLE NICOLE JOAQUIN

THESIS

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Adviser:

Professor K. Jimmy Hsia
ABSTRACT

This thesis explores the effects of mechanical stimuli on cell patterning and behavior, which may influence our understanding of in vivo processes such as morphogenesis, wound healing, disease progression, tumor formation, and metastatic events. We begin by looking at the effects of a stiffness gradient in three-dimensional culture environment, which builds upon prior research on the effects of stiffness in a two-dimensional culture, but provides a platform for studying these outcomes that is more akin to the in vivo environment. Then we look at the effect of hydrostatic pressure using a non-contact pressure chamber that provides a uniform pressure environment while maintaining physiological conditions needed for cell growth. By seeking to better understand the migratory responses of cells to various mechanical stimuli, we gain tools and knowledge that can be used to aid in diagnoses and treatment of a variety of diseases.
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CHAPTER 1: INTRODUCTION

1.1 Mechanobiology

1.1.1 Cell Response to External Stimuli

Cells, like all living things, have the ability to gather cues from their environment and respond to them in a wide variety of ways. This is an important capability, essential for many *in vivo* processes including morphogenesis, growth, disease progression, wound healing, and numerous other adaptations to natural stresses put on the body. Cells take in information from thousands of cues at a time, producing a unified response that allows them to grow, evolve, move, and survive. This activates a cellular feedback loop, without which our bodies would be unable to regulate many processes that are so essential to our survival.

One widely cited work that exemplifies cells’ ability to respond to cues reveals the ability of a neutrophil to maneuver in response to localized release of a chemoattractant nearby (Servant et al. 2000). In this work’s famous timelapse videos, a neutrophil rapidly polarizes in the direction of a chemoattractant released into the media, causing the cell to migrate in that direction with very little lag time. Although the signaling and responses that take place in the body are typically less extreme, similar effects can certainly be observed *in vivo*. 

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A cell’s ability to respond extends not just to biochemical cues, but also to a wide range of mechanical stimuli. These typically relate to force (tension, compression, shear, pressure), or to geometric cues (curvature, shape, etc). In either case, there is interplay between the cell’s structural components, called the cytoskeleton, and the surrounding physical environment, which results in these changes within the cell. The change or response is termed mechanotransduction, and it is manifested through altered biochemical pathways taken within the cell which direct the cell’s physical response. At any given time, cellular organelles and the cytoskeletal components can be thought of as being in a delicate balance, and any extrinsic perturbation requires the intracellular components to “react” to restore a new balance. In doing so they may bring about other changes on themselves or their surroundings.

1.1.2 Mechanotransduction pathways

The underlying mechanisms by which cells sense and respond to their mechanical surroundings are widely studied today. In most cases, the mechanical signal is eventually converted to a chemical signal which can enact a change. Several mechanisms have been suggested, and they typically cite physical shifts in various conformation-dependent steps within a biochemical pathway. Many of these shifts are actuated on the cell membrane, where the cell is most exposed to the external environment and therefore subjected to the most extrinsic factors. Forces can then be transmitted into signals inside the membrane which result in altered pathways
(Mammoto et al. 2013). For example, a shear force may cause an ion channel within the cell membrane to open, allowing an unnatural concentration of that ion within the cell. The ion, in turn, may up- or down-regulate the process or processes it controls.

Geometry, too, can have profound effects on the fate of a cell. Small changes in geometric features can alter migration, proliferation, or cell-cell signaling, and can even be the difference between life and death (Chen et al. 1997). In a study by Chen and colleagues, endothelial cells could be induced to undergo proliferation or apoptosis, simply by changing the shape and size of the surfaces to which they could attach. Small “islands” with adhesion molecules were created with different shapes and sizes, and cells that were adhered to the smallest islands eventually underwent apoptosis. Cells with the most area to spread, on the other hand, proliferated most rapidly.

Numerous other examples of cell mechanotransduction are available for study. Many are much more complex than the two examples given above, and are often processed within the nucleus. A uniaxial compression force, for instance, could perturb the physical balance of the cytoskeleton, the structural unit of the cell. Since the cell’s organelles are interconnected through these cytoskeletal elements, this transient change may be amplified in the nucleus, causing a significantly altered chain of events. The nucleus can then enact the appropriate changes in cell size,
shape, motility, or even fate.

1.1.3 Physiological Relevance

The true in vivo environment of a cell is a compilation of thousands of extracellular cues, integrated to form a unified response. Although the opening of a single ion channel may not be enough to induce a dramatic response from the cell, the individual signals received within the nucleus can be integrated over time and space, so that the magnitude of the response may be proportional to the strength and/or persistence of the signal. Through this mechanism, cells can respond in a unified fashion to significant cues, while more or less ignoring those cues that have little bearing on their overall state. The total response takes into account both mechanical and chemical cues in the environment, and can occur over a range of time scales, from a few milliseconds to several days or even years.

It is also important to note that cells communicate with one another via signals- the same variety of signals that they sense from their extracellular environment. In doing so, they may act in a collective sense to enact changes on the tissue or even organ level. Cells can also act communally in vitro (Grigola et al. 2014). For example, cells can develop into masses and then migrate collectively, even taking on specific roles within these masses, namely the leader (“tip”) and follower (“stalk”) cells (Rørth 2012). This collectivism is directly related to in vivo processes, such as wound healing, in which the same cell hierarchy forms as cells migrate in order to
close a wound. Cells nearest the wound become tip cells, and promote proliferation and migration toward the open wound until it is closed. This collectivism enables a more direct response at the targeted site. A similar collaborative effect occurs in other physiologically relevant processes, such as cancer cell migration, growth, and morphogenesis.

Even when cells are not migrating, cell-cell communication is essential to proper organ and tissue function. Organs rely on the functions of many specialized types of cells, which must communicate with each other in order to properly perform their duties. The same chemical and mechanical signaling mechanisms that cells use to migrate collectively can also be used to enable these tissues and organs to function. Thus, all cells must not only be able to respond to chemical and mechanical signals, but also produce them.

1.2 Research Motivation

The study of mechanobiology is emerging as a more and more relevant factor in many aspects of cell development. Once underestimated for its ability to affect changes in the cell, it is now clear that mechanics have a powerful impact on cell, tissue, and organ function (Mammoto et al. 2013, Lo et al. 2000). The field is highly interdisciplinary, relying on an understanding of both mechanics and biology, and
how one might apply to the other. Despite its challenges, gaining a better understanding of these applications can have profound impacts on medicine and our understanding of the natural world. Cancer, hypertension, arthritis, and sickle cell anemia are just a few examples of diseases which are caused by mechanical deviations from normal physiology (Carey et al. 2012, Wang et al. 2013, Peera et al. 2010). Understanding what causes pathological changes may provide important insights into how they can be repaired. For example, mechanical stimulation is a largely unexplored, yet very viable realm of treatment for these and other conditions. Furthermore, a better understanding of “normal” mechanical behaviors is a crucial step toward the development of artificial tissues and/or other types of regenerative medicine.

1.3 Thesis Topics

This thesis is organized into two body sections, each dealing with a different aspect of a cell’s mechanical environment. Chapter 2 discusses the effect of stiffness in a three-dimensional gel. It examines the results of plating breast epithelial cells on a controlled anisotropic stiffness gradient created by overlaying fabricated sinusoidal PDMS patterns with a layer of gel. The question of how gel effective stiffness influences cell behavior is motivated by studies demonstrating that cells modulate their shape, fate, and migration patterns depending on the stiffness of the two-dimensional substrate on which they reside. Demonstrating the ability to produce
similar results in a three-dimensional gel is therefore a topic of significant interest, since these conditions better replicate the environment found in vivo.

Next, Chapter 3 deals with the effects of hydrostatic pressure on cells. Cancer cells in vivo are known to experience elevated levels of interstitial pressure due to a buildup of fluid from leaky vessels and poor lymphatic drainage (Lunt et al. 2008). However, it is still not certain whether this pressure is at all responsible for, or a consequence of, cancer, or if the two are even related. The goal of this section is to better understand this relationship, and determine if any signs of malignancy can be induced in normal mammary epithelial cells by means of hydrostatic pressure.
CHAPTER 2: THREE-DIMENSIONAL STIFFNESS GRADIENT EFFECT

2.1 Introduction

2.1.1 Tissue Engineering Overview

With obesity, heart disease, and similar disorders gaining prevalence around the world, the number of patients with irreparable organ or tissue dysfunction increases rapidly every year. Organ and tissue transplants from donor sources have a high success rate for most patients, but the availability of viable transplant organs is severely lagging behind demand, resulting in a long, growing list of unfulfilled transplants (2011 Annual Report of the U.S. Organ Procurement and Transplantation Network). As a result, regenerative medicine has emerged within the past three decades to provide alternative methods for restoring organ and tissue functions essential to life without the need for donors (Lavik and Langer 2004; Berthiaume et al. 2011).

These approaches aim to restore the function of a vital organ by either repairing the disabled portions of the current organ or replacing the organ entirely with an engineered tissue construct. Variability from patient to patient presents a significant challenge in regenerative medicine, and thus tissue engineering has
demonstrated the most promise. Instead of trying to repair dysfunctional cells, tissue engineers attempt to transform healthy cells, taken either from another source within the patient’s body (autograft) or another donor (allograft), into new functional tissues by culturing them outside the body under the proper conditions.

Nevertheless, to “engineer” more complex tissue structures with the precise properties of their in vivo equivalents presents a wide array of challenges. Within the past 10-20 years, numerous researchers have investigated ways of recreating tissue function from various cells, often by developing complex substrates that contain well-positioned chemical factors and complex structures to ensure that the cells grow, divide, and differentiate as planned. Much progress has already been made in this field, and it is now quite commonplace for burn victims or injured athletes to receive tissue-engineered skin or muscle grafts made from cells taken from their own body. However, successes in this field have primarily been limited to implants with simplistic components and functions.

One key challenge that limits the ability to create more complex structures is the need to provide nearly immediate nutrient supply to implanted tissues. Cells that do not have a supply of essential nutrients cannot survive for more than an hour or two at best. In thin tissues, such as skin or cartilage, diffusion may be sufficient to provide the nutrients needed to sustain the tissue until more reliable sources are available. However, in more complex tissues or organs, there is a low probability
that a tissue will survive past its initial transplantation if the thickness of the implant exceeds the diffusive limit of nutrients through the layers of cells (Laschke and Menger 2012). Thus, an important current challenge in tissue engineering is finding a way to initiate blood flow to the implanted tissue during or shortly after surgery.

To create blood flow in vivo, there are two options that tissue engineers can consider: angiogenesis and inosculuation (Fischer et al. 2012). Angiogenesis requires new blood vessels to form in the tissue in vivo after the transplantation surgery; inosculuation, on the other hand, begins with tissues that already contain microvascular networks, and then facilitates the linking of these networks with existing vasculature. These two processes can be seen in Figure 1.1, below.

Figure 1.1: Image showing the angiogenesis (left) and inosculuation (right) processes. Angiogenesis requires new blood vessels to form in the tissue, while inosculuation only requires the existing vessels to join with preformed vasculature in the tissue (Laschke et al. 2012).
Angiogenesis, which requires new vessels to form, is a very slow process on its own. Blood vessels only grow in vivo at a rate of about 5 μm/hr (Orr et al. 2003), so it would take approximately 20,000 hours, or >1 year for blood vessels to naturally reach the center of an organ 10 cm in diameter. Even with the inclusion of factors to speed up this process, it is unlikely that vascularization could be made speedy enough to sustain large tissues. Thus, most researchers have turned to inosculation, which shows more promise as a vascularization technique (Laschke et al. 2012). With this method, pre-built vascular networks only need to connect to the existing vasculature within the body before they can begin to function, taking much less time than forming all new networks.

Although it allows better and more sustained growth of tissues, inosculation adds a new layer of complexity to tissue engineering. Not only must the implants become fully functional replacements for disabled organs, but they must have vascular structures embedded into the assembly. Moreover, these structures must contain lumina, or vacant inner passageways, which allow nutrient-rich fluid to flow through. Ideally, these vascular structures would be surrounded with smooth muscle cells which would contract and relax the vessels and encourage the flow of nutrients. These challenges are still ongoing, and work in tissue engineering and cellular mechanics have tried to address these problems.

The advances that have been made to date in the field of tissue engineering would not have been possible without an appreciation of the importance of
mechanotransduction in cellular growth and development. Self-assembly of cellular constructs \textit{in vivo} during morphogenesis depends largely on the physical, as well as chemical, environment, and these requirements can be exploited to create similar constructs outside the body. Nevertheless, in order to address current and future hurdles in tissue engineering, including vascularization of tissues, we must first gain a comprehensive understanding of cell migration, aggregation, and organization to determine how external cues can direct these processes. Once these issues are well-understood, tackling the complex spatial issues related to tissue engineering will be much more feasible.

2.1.2 Cells and Stiffness

Tissues \textit{in vivo} are made up of cells and extracellular matrix. This matrix is a composition of water, proteins, polysaccharides, fibers, and small molecules that support the cells by providing stability, structure, communication, attachment sites, and many other functions. Importantly, the composition of the extracellular matrix varies drastically from one region of the body to another, and tissues gain many of their bulk mechanical properties from these differences. For example, while breast tissue has a bulk elasticity in the range of hundreds of Pascals, bone can be as stiff as 2-4 GPa (Figure 2.1). Thus, mechanical stimuli are ubiquitous within the \textit{in vivo} environment.
Most cells are considered adherent, meaning that they must attach to a surface in order to survive. Attachment occurs at distinct locations, called focal adhesions, and it is at these sites that cells can probe their underlying surface, an important component of their environment. One mechanism through which this mechanotransduction could occur is through passive tension in the cell’s focal adhesions. For example, cells’ adhesions may each exert a specified amount of force, and a localized biochemical response could occur depending on the level of displacement this force can produce.

Physical considerations were overlooked for a number of years, despite the evidence that the in vivo environment contained an array of mechanical cues giving rise to various pathologies. Cells were grown on functionalized plastic or glass dishes with elasticities upwards of 100 kPa, far from the cells’ native tissues. This approach held until late in the 20th century. Finally, cross-functional research teams began to investigate these effects with a variety of studies, giving rise to the field of

Figure 2.1: Range of in vivo cellular microenvironment elasticities for different types of tissues (Weaver et al. 2009).
mechanobiology. In an early study to probe the effect of stiffness on cell behavior and physiology, Pelham and Wang used polyacrylamide gels to alter stiffness without changing chemical composition or ligand density, finding that fibroblasts and epithelial cells on soft substrates exhibited focal adhesions that were irregular and dynamic compared to those on firm substrates (Pelham and Wang 2007).

This initial study changed the way that biologists looked at cells and spurred a number of new questions related to stiffness effects. Among the studies to investigate these questions early on, Lo and colleagues were the first to implicate substrate rigidity in cell migratory behaviors (Lo et al. 2000). They created a gradient in substrate stiffness over a short distance by placing two drops of polyacrylamide with different mixing ratios under a coverslip, allowing only a small amount of mixing at the interface. 3T3 fibroblast cells were then added sparsely on top, and after 15 hours, these cells’ migration patterns were recorded. Cells were observed to readily cross the “boundary” toward the stiff gel, but were unsuccessful at crossing in the opposite direction. Furthermore, upon the addition of tension forces on the substrate, cells migrated toward the applied tension, indicating that the mechanism for stiffness response may be related to the amount of tension or displacement cells “feel” at their lamellipodia.

Since these landmark studies, several others have looked at stiffness effects on cells and observed trends which have implications on a range of cell processes, behaviors, and morphological features (Isenberg et al 2009, Kidoaki et al. 2008,
Yeung et al. 2005, Gray et al. 2003). For example, cells placed on substrates of different stiffness often adapt their own structural rigidity to better match the surface (Ghosh et al. 2007, Solon et al. 2007). Effects on proliferation rate (Peyton et al. 2006), differentiation outcomes for stem cells and other progenitor cells (Ren et al. 2008, Evans et al. 2009), and other biochemical and physical processes have also been documented. These outcomes are not limited to fibroblasts and epithelial cells; many other cell types have also been shown to respond to stiffness, although their responses are not always predicted by the outcomes seen in fibroblasts or other cell types (Isenberg et al. 2009, Yeung et al. 2005, Deroanne et al. 2001).

2.1.3 Three-Dimensional Stiffness Motivation

The work described in Section 2.1.2 was performed in the context of essentially two-dimensional culture conditions. Polyacrylamide gel, typically the material of choice for these stiffness applications, is a polymer with a tunable elasticity (.5 kPa-50 kPa), making it convenient for changing stiffness without altering any other parameters. However, these gels are synthetic, so they do not contain any of the components of in vivo extracellular matrix. Cells in vivo communicate, migrate, and assemble by remodeling and rearranging fibers in the ECM. Although polyacrylamide gels can be functionalized with similar proteins and fibers, the adhesion sites available are limited to one surface of the cell, causing the cells to take on a 2D morphology similar to that of cells on glass or a petri dish (Baker and Chen 2012). Therefore, these polyacrylamide gels do not closely replicate physiological conditions. Gels that are capable of being remodeled, transmitting
cellular forces, and providing dynamic adhesion sites, are much more physiologically relevant in experiments (Pedersen and Swartz 2005) and have largely replaced these two-dimensional systems in recent years. Thus, it is necessary to consider effects of stiffness in a truly “three-dimensional” sense.

Controlling stiffness in three-dimensions without modulating any physical or chemical properties which significantly alter cell behavior, such as ligand density, fiber density, or porosity, presents a significant hurdle (Hayen et al. 1999). In one study, Hadjipanayi demonstrated 3D durotaxis by creating wedges of collagen matrix, and then compressing them to a uniform thickness, giving the final matrix a constantly increasing stiffness gradient (Hadjipanayi et al. 2009). Although this method provides a final substrate with a linearly increasing stiffness, the process of compressing the wedges also alters the density of fibers within the matrix, which could have induced migration. Stiffness effects have been shown to have unique interplay with other biochemical and physical characteristics (Stroka and Espinosa 2009, Rodriguez and Schneider 2013), and thus it is highly desirable to alter stiffness without changing other physical characteristics of the gel.

2.1.4 Overview

The method we’ve developed uses Matrigel™, a reconstituted basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma, which is composed primarily of laminin and other matrix proteins, including collagen IV, proteoglycans, and entactin. Rather than altering the bulk material properties of the gel, we instead
control the “effective stiffness” of the gel at the surface with an underlying geometry. This idea is demonstrated in Figure 2.2.

Figure 2.2: Illustration showing the difference in effective stiffness on a thin gel compared to thick gels. The displacement on the surface of a thin gel due to a traction force is much smaller than that of the same force on a thick gel.

This concept relies on the use of an underlying substrate that is much more rigid than the Matrigel™ membrane. Overlaying the cells on soft gel atop an underlying rigid substrate causes them to “feel” a different effective stiffness depending on gel thickness. Evidence supports the idea that durotaxis is caused by overall resistance felt by lamellipodia, rather than a bulk material stiffness. For example, cells exhibit different behaviors on micropillars of different lengths, even though the size and material characteristics of the pillars remained unchanged (Saez et al. 2005). This suggests that cell’s behavior may be controlled by a so-called “effective stiffness”, or spring constant, rather than the substrate material’s elastic modulus.

A few studies have documented cellular responses to effective stiffness. In 2009, Cortese and colleagues used highly crosslinked PDMS with indented patterns, and
attached a thin layer of soft PDMS on top, creating an effective stiffness difference between the regions above the indents and those above the bulk PDMS (Cortese et al. 2009). Cells on the top layer of PDMS migrated toward the stiffer regions supported by the thicker PDMS pattern. In a similar study, Kuo and colleagues later used polystyrene beads, steps, and grooves underneath a soft polyacrylamide gel, yielding similar results (Kuo et al. 2012). Further investigating these effects, Buxboim and colleagues looked at a range of gel thicknesses with underlying features, and found that the range of cell “feeling” extended to about 10-20 \( \mu m \) in depth when using polyacrylamide gels of mismatched elastic modulus (Buxboim et al. 2010).

The studies described above help demonstrate that the stiffness sensing mechanism used by cells is controlled by effective stiffness, rather than an inherent elastic modulus. These findings have produced a new realm of possibilities for manipulating cells in tissue engineering and biology. Nevertheless, these studies have all looked at soft gels that are biologically inert, such as polyacrylamide gels and soft polydimethylsiloxane (PDMS). Here, we extend these works by demonstrating the application of this technique to Matrigel\textsuperscript{™}, a soft basement membrane gel (E\( \approx \)300 Pa) which gives cells the opportunity to remodel and rearrange the fibers as they would in vivo. By overlaying geometrically patterned PDMS substrates with soft Matrigel\textsuperscript{™}, we produce gels which closely replicate in vivo conditions with continuous stiffness gradients on their surface. We then characterize the stiffness profile of the gels using the Finite Element Modeling
software ABAQUS/Standard, and speculate on how the stiffness and stiffness gradient might relate to cell migration.

2.2 Materials and Methods

2.2.1 Cell Types

We study four types of cells in this section: MCF10A, MDA-MB-231, HUVEC, and C2C12 cells. MCF10A cells (ATCC® CRL-10317™) are a non-tumorigenic mammary gland epithelial cell line derived from a 36-year-old Caucasian female. MDA-MB-231 (ATCC® HTB-26) are analogous mammary gland adenocarcinoma epithelial cells derived from a metastatic cancer site of a 51-year-old Caucasian female. The HUVEC cell line (ATCC® CRL-1730) is an endothelial cell line derived from human umbilical vein tissue. C2C12 cells are a cell line of myoblasts derived from a two month old female C3H mouse. These cells differentiate well into myotubes with the proper culture media and conditions. All cell lines mentioned in this section are immortalized and adherent, meaning they require attachment to a surface in order to survive.

2.2.2 Cell Culture

Prior to experiments, all cell types were maintained in monolayer culture on plastic tissue-culture treated dishes at 37°C and 5% CO₂. Each cell line was treated with different media and supplements. MCF 10A cells were cultured in 50/50 Dulbecco’s
Modified Eagle’s Medium/Ham’s F12 Nutrient Mixture (DMEM/F12) with the following supplements: 5% Horse Serum, 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 5 U/ml penicillin, 100 µg/ml streptomycin. Human Umbilical Vein Epithelial Cells (Invitrogen) were cultured in Medium 200 (Invitrogen) supplemented with 2% fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. MDA-MB-231 breast adenocarcinoma cells were cultured in Leibovitz’s L-15 Medium with 10% fetal bovine serum. Finally, C2C12 mouse myoblasts were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. During all time lapse videos, cells were maintained in a humidified environment-controlled cell chamber (37°C, 5% CO₂).

2.2.3 Wavy Patterns

In the initial experiments, we used wavy patterns made from PDMS and overlaid with Matrigel™ to demonstrate control of cells on reconstructed basement membrane material on the basis of surface effective stiffness. Approximately sinusoidal wavy pattern molds were generated with a buckling technique based on the concept of strain mismatch that does not require the use of cleanroom facilities (Jiang et al. 2007). Thin films of PDMS (Sylgard 184, Dow Corning) were created by spin coating and then cured. After the films cure, a thicker strip of PDMS is placed in the uniaxial straining device (Figure 2.3a) and strained to a given percentage. Both the unstrained thin film and the thicker strip are plasma oxidized, and then bonded.
firmly together. After four hours, the strain in the thick strip is released, causing the thin film to buckle locally to form uniform sinusoidal waves. In order to achieve these patterns, the thin film must be sufficiently stiff relative to the thick slab of PDMS, which is ensured by either increasing the ratio of base to crosslinker or increasing curing temperature. The thickness should also be much smaller than the thick strip. The thickness of each PDMS piece, crosslinking ratio, and the strain percentage are parameters that can be varied to create the desired pattern dimensions.

Figure 2.3: (a) An image of the uniaxial straining device used to create wavy patterns. A thick slab of PDMS is secured under the four bolts. The knob is turned until it reaches a certain level of displacement. The slab and a thin film of PDMS are oxidized and bonded together while the slab is still outstretched. When the strain is released, the thin film buckles due to strain mismatch. (b) Images of a PDMS wavy pattern replica ($\lambda = 1400 \mu m$, $A = 350 \mu m$).
The dimensions of the pattern can be found from Equation 1, below:

\[ H(x, y) = A \sin\left(\frac{2\pi x}{\lambda}\right) \]  \hspace{1cm} \text{Eqn. 1}

where \( \lambda \) and \( A \) are the wavelength and amplitude of the pattern. Once the sinusoidal films have been formed, they are set in PDMS to create molds, from which numerous PDMS copies can be generated. Figure 2.3b shows a few copies of the patterns, on top of which Matrigel™ and cells would be plated.

2.2.4 Plating procedures

Matrigel™ is a unique biological gel that is a liquid at 4°C, but gels quickly at room temperature. Thus, all pipette tips and patterns must be chilled prior to use. During plating, PDMS wavy patterns are set on ice and then overlaid with a thin layer of Matrigel™ (approximately 60% laminin, 30% collagen IV, and 8% entactin) to just cover the wave peaks. The most effective way to ensure that excess Matrigel™ is not used is to pipette small amounts of gel onto the surface at a time and spread the gel as thinly as possible before pipetting more gel. Confirmation that the peaks are fully covered can be made by visual inspection. Once this is done, patterns are returned to 4°C so the Matrigel™ can reliquify. This helps ensure a smooth and even gel surface. After a minimum of one hour at 4°C, plates are moved to the 37°C incubator to initiate Matrigel™ gelation. Gelation takes approximately 15 minutes. After 45-60 minutes the gel becomes dried out, so care should be taken to plate cells within the 15-45 minute window. This can be done by beginning to trypsinize the cells at
approximately the same time that the Matrigel™ plates are moved to the incubator. Cells are lifted from culture dishes using 2mL of TRYP LE (Invitrogen), centrifuged at 100 g, and then resuspended in minimal growth medium and added to the surfaces of the gels at a density of 50,000 cells/cm$^2$ (Lee et al. 2007).

After cells are added to the surface, the patterns should be returned once again to the incubator until cells are settled to the surface of the gel and are no longer floating in media (approximately 10 minutes). After the cells attach, the media is replaced with growth media containing 2% Matrigel™, and plates are stored at 37°C, 5% CO$_2$. Figure 2.4 summarizes the entire fabrication and plating process.

Figure 2.4: Entire fabrication and plating process. In the first step, a sinusoidal mold is created by buckling of a PDMS thin film. Next, numerous copies are made from the mold. These pattern copies are overlaid with Matrigel™, and the gel is allowed to solidify at 37°C. Finally, a cell suspension is added to the top of the gel, and cells embed a few μm within the Matrigel™.
2.2.5 Scanning Electron Microscopy

In order to verify that the “3D On Top” protocol described by Lee and colleagues created an environment that allowed the cells to take on a three-dimensional morphology, we performed Scanning Electron Microscopy of cells plated on a flat dish with the 3D On Top procedure described in Lee, et al. Prior to imaging, cells were fixed for 4 hours at 4°C using 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Na-Cacodylate buffer. The samples were then rinsed twice with 0.1 M Na-Cacodylate buffer, dehydrated using subsequent 10 minute rinses of 37%, 67%, 95%, and 100% (3x) ethanol, and then a critical point drying procedure was performed using a Samdri-PVT-3D (Tousimis). The sample was then sputter coated with gold palladium using a Denton Desk II TSC turbo-pumped sputter coater and then visualized with a Philips XL30 Environmental Scanning Electron Microscope operating in Hi-VAC mode at 5 kV.

2.2.6 Cell Velocity Analysis from Timelapse Image Sequences

Cell velocity was found with a C++ program developed by Michael Grigola. The program prompts the user to identify cells or cell clusters in the first frame to be tracked. Each grouping is then identified by key points, which are tracked throughout the video. Key points are weighted by their proximity to the center of the cell, closeness to the original orientation, and how close they match the previous frame (Grigola and Hsia 2014). The center of the cell is found by searching near the key points for corners or edges, and then finding the center. Once the center of each
cell is determined in every frame, cell velocity is computed in MATLAB based on displacement over a given time. Finally, the image is vertically “binned” and the average velocity within each bin is calculated to find the velocity profile of cells moving over the surface of the gel.

2.2.7 Finite Element Modeling

For each geometry, we used ABAQUS/Standard (Providence, RI) to produce representative models of the layer of Matrigel™ that lies complementary to the pattern surface. The Matrigel™ was modeled as a linearly elastic, homogeneous, and isotropic material with an elastic modulus of 300 Pa (Wood et al. 2010). For simplification, Matrigel™ viscoelastic behavior was not considered, since the measurement of effective stiffness is an instantaneous effect (Rao et al. 2012). The PDMS-Matrigel™ interface was modeled as a fixed boundary on the bottom of the gel with motion restricted in x, y, and z (Figure 2.5).

Cells are known to apply forces at individual focal adhesion sites, rather than along the entire bottom surface of the cell. However, applying a point force in ABAQUS is not reasonable, and focal adhesions are several orders of magnitude smaller than the size of the wavy patterns. Therefore, partitioning the Matrigel™ surface into regions at this length scale without sacrificing mesh quality proved prohibitively computationally expensive. Since we are interested in a qualitative, rather than a quantitative measure of the stiffness, tractions were instead applied uniformly to
small square regions on the length scale of the entire cell, where mesh quality could be made sufficiently high. Figure 2.5 shows a sample of this force application at one location. These traction regions were applied and analyzed one by one, moving across the surface of the gel along the x-axis. Each incremental traction region was applied during a different static simulation. Local surface displacement was then extracted at the center of each traction region and used to determine the spring constant, $k$, of the surface as a function of location along the gel surface.

![Finite element simulation example](image)

Figure 2.5: Finite element simulation example where force is applied to a small region on the surface of the gel. The displacement is then measured at the center of the region, and effective stiffness, or spring constant, is calculated.

### 2.2.8 Mesh Convergence and Solution Verification

Mesh convergence was ensured using an increasing number of total nodes, starting at 34,028. The applied force was kept constant, and the displacement at regions along the surface of the model above the peak, valley, and two midpoints were measured. The resultant convergence plot can be seen in Figure 2.6.
Convergence was also checked for the number of elements in the traction region, holding the total number roughly constant. We also verified the finite element mesh analytically by confirming that relationships between the displacement solutions for different levels of force were consistent with the Boussinesq solution (Boussinesq 1885) for a traction applied at the center of a flat plate thick enough to be considered semi-infinite, as previously demonstrated (Maloney et al. 2008).

Figure 2.6: Mesh convergence results for the four regions sampled (regions above the peak, valley, and two halfway points).

2.3 Results

2.3.1 Scanning Electron Microscopy
Figure 2.7, below, shows several images taken with scanning electron microscopy. Note that the morphology of the cells is relatively spherical, with filopodia protruding from the cell. This contrasts with the so-called cobblestone morphology of cells on a flat plate with no extracellular matrix (Figure 2.8). Although cells in the 3D On Top protocol are placed on top of the gel, they appear to partially embed themselves into the gel, with fibers from the extracellular matrix covering most of the cell surfaces, indicating an inherently “three-dimensional” morphology (Baker and Chen 2012).

![Figure 2.7: Scanning electron microscopy images of MCF10a cells “embedded” on the surface of the 3D gel. These images illustrate the 3D nature of the 3D-On Top plating method used in these experiments.](image-url)
2.3.2 Cell Sensitivity to Changes in Thickness

On the wavy patterns, MCF10a cells were observed to migrate directionally toward a midline to form linear cell clusters. This result can be seen in the timelapse image series in Figure 2.9.

By cross-sectioning the gels and imaging under phase contrast microscopy, we can also observe that the cells indeed migrate in the direction of minimum thickness, which is also toward the positive stiffness gradient. Evidence of this observation can be seen in Figure 2.10.
Figure 2.9: MCF10a 24-hour timelapse series after random dispersion of cells at t=0. Over the course of 24 hours, cells aggregate into a cluster above the peaks of the patterns, where gel thickness is lowest, and therefore the effective stiffness is at its highest.

Figure 2.10: Cross-sectional image showing MCF10a cell locations in the gel. Due to the small gel thickness above the peaks, cells are drawn to these regions and form linear aggregates at periodic spacing along the pattern, as seen in Figure 2.9, above.
MCF10a cells on top of a relatively thin gel layer (10s of μm’s) exhibited persistent and repeatable migration toward the thinner portions of the gel. However, consistent with previous reports (Kuo et al. 2012; Buxboim et al. 2010), gel height did not bias cell migration when the gel was very thick, as seen in Figure 2.11.

Furthermore, Figure 2.9 shows that cells on large patterns only migrate if they are near enough to the peaks, resulting in columns of cells with intermittent unaligned cells between the peaks. However, on the smaller wavy patterns, cell migration toward the thinner gel region resulted in aligned columns of cells above each of the peaks, with few or no cells above the valleys. This result can be seen in Figure 2.12.

Figure 2.11: Image of random MCF10a cell dispersion observed when gel volume is doubled from that of Figure 2.9.
2.3.3 Simulation Results

ABAQUS/Standard results show that the effective stiffness of the Matrigel™ surface is dependent on the thickness of the gel layer, although it is not a linear dependence. As expected, a force applied on the surface of a thin gel is influenced much more by the fixed boundary of the PDMS than that on a thick gel. Typical stiffness and stiffness gradient profiles for the surface of the Matrigel™ can be seen in Figure 2.13 for wavy patterns.

Using stiffness gradient data found in ABAQUS, we were able to obtain the relationship among the gel height, slope, and stiffness gradient for Matrigel surfaces. This relationship can be seen in Figure 2.14. By finding the plane of best fit and solving the differential equation, this information can be used to help develop
surfaces with constant stiffness gradient. Cells plated on this type of surface should, in theory, have a uniform, non-zero cell velocity.

Figure 2.13: Wavy Pattern stiffness and stiffness gradient profiles, with wave peaks occurring at 0% and 100%. As expected, stiffness is at its highest above the peaks, and lowest above the valleys. Cell migration toward the peaks occurs within approximately 25% of this wave peak, which is where the magnitude of the stiffness gradient is at its highest.

Figure 2.14: Relationships among gel height, surface slope, and stiffness gradient.
2.3.4 Cell Migration Velocity

We used time-lapse phase contrast microscopy to record cells plated on Matrigel™ atop the wavy patterns for 24 hours after seeding. From these videos, it appeared as though cells’ velocity was increasing as they neared the thin regions. We analyzed the horizontal velocity of the cells as they approached these region for several videos. On the wavy patterns, the reference point is the peak of the pattern (where the gel height is at a minimum). Typical velocity profiles of cells on the wavy patterns and a correlation analysis with the stiffness gradient profile can be seen in Figure 2.15.

![Figure 2.15: Velocity profile of MCF10a cells on 1400 μm pattern (top), with the left side of the figure indicating the peak of the pattern. A correlation analysis was performed to determine the relationship with the stiffness gradient profile on the surface (bottom).](image-url)
2.3.5 Linearly Graded Matrigel Samples

In order to analyze the relationship between stiffness and cell migration velocity, linearly graded gels were used with the techniques previously mentioned. This allowed us to accurately determine the thickness of the gel at any given point based on the location of the start of the gel ($h = 0 \mu m$) and the known slopes of the underlying PDMS substrate. Cells were again observed to migrate toward the stiffest section, i.e. the edge of the gel. Several slopes were tested ($m=0.1, 0.5, 0.8, 1.0$). A typical stiffness and stiffness gradient profile of these gels, found in ABAQUS, is shown below in Figure 2.16.

![Stiffness and Stiffness Gradient Profiles](image)

**Figure 2.16**: Stiffness and stiffness gradient profiles for linearly sloped PDMS wedges. Again, the highest stiffness and stiffness gradient occurs at the thinnest portion of the pattern.

Cell velocity was also analyzed on these slopes, but wide variability was observed in the velocity patterns and magnitudes, and results are so far inconclusive. A few
experiments showed relative velocity correlation with stiffness gradient. One such preliminary result can be seen in Figure 2.17.

Figure 2.17: Velocity profile of cells on wedges (blue) overlaid on surface stiffness gradient profile plot (red). \( \lambda = 1400 \ \mu m, A = 350 \ \mu m \)

2.3.6 Cell Types

Although the majority of experiments in this work were performed using MCF10a cells, the wavy patterns were also tested with other cell types. Endothelial cells (Human Umbilical Vein Endothelial Cells), undifferentiated mouse myoblasts (C2C12), and malignant human mammary adenocarcinoma cells (MDA-MB-231) were all plated on the wavy pattern gels with random dispersion at 50,000 cells/cm². Each cell line demonstrated migration and alignment on the patterns, although each had a different final morphology. These results can be seen in Figure 2.18.
Figure 2.18: Different cell types plated on wavy patterns. Morphology differs greatly from one type to another, but the behavior is similar. Cells pictured above are A) HUVEC, B) MCF10a, C) C2C12, and D) MDA-MB-231 cells.

2.4 Discussion

In this study, we have demonstrated that cells in three-dimensional culture respond to “effective stiffness” based on the geometric constraints of the gel, rather than simply bulk properties. Other groups have reported similar effects due to the thickness of an inert hydrogel, but to our knowledge we are the first to use a biological substrate as a means of controlling effective stiffness without changing gel properties, and to analyze the cell migration speed on these patterns. We demonstrate the ability to aggregate cells into specific structures using the wavy
patterns, providing a new experimental platform for applications such as tissue engineering, where it is essential to have fine control of cell migration in order to form precisely positioned structures.

An interesting, though preliminary, result is the relationship between cell velocity and the stiffness gradient of the surface. The mean velocity of cells as they pass through various positions on the surface appears to scale linearly with the stiffness gradient at those positions. This indicates that the stiffness gradient, and not the stiffness itself, may be responsible for cells' migratory behavior. This finding is supported by the notion that the cell sensing mechanism responsible for creating durotaxis could be related to differences in the feedback felt at opposing sides of the cell in response to fluctuating tensional force. Although the mechanical linkage to directed cell migration still is unclear, it has been suggested that there may be a stiffness-dependent mechanism by which transient focal adhesions can be reinforced, prolonging their release and causing the cells to preferentially migrate toward the higher stiffness (Plotnikov and Waterman 2013). By this theory, it is plausible that greater differences in stiffness between the opposing ends of a cell would yield faster motion.

Another theory is that these forces applied at these focal adhesions result in varying levels of displacement at stretch-mediated Ca$^{2+}$ channels (Mammoto et al. 2013). These differences could result in localized release of Ca$^{2+}$ ions, which induces a local contraction response. On a high stiffness gradient, contraction frequency would be
much higher on one side of the cell than the other. Integrating these differences over the entire edge of the cell could result in a highly biased migratory response. Furthermore, the bias in contraction and therefore migration could again depend on the quantitative difference in stiffness from one side of the cell to the other, resulting in a linear response between stiffness gradient and migration speed.

Despite some evidence that the velocity and stiffness gradient have a linear dependence, we acknowledge that there may be multiple factors at play causing the cells to appear aligned on the wavy patterns. In addition to the durotaxis effect we are seeing, it is likely that an additional mechanism is causing cells to align above the wave peaks. Cells that arrive at the center of the peaks early in the 24 hour sequence generally move very slowly because they no longer sense differences in stiffness. Despite their immobility, these cells constantly exert tension force on the surrounding gel through their focal adhesions. These tension forces can then be transmitted through the gel at length scales much higher than the size of the cell itself (Guo et al. 2012). The result is that unaligned cells in the surrounding gel would experience a maximal tension force in the direction of the peaks. This would provide a secondary mechanism, called plithotaxis, by which the cells could migrate toward the peaks (Roca-Cusachs et al. 2013). Plithotaxis is typically a collective effect (Tambe et al. 2011), and Figure 2.19 shows that on the wavy patterns, cells often congregate locally into acini prior to migrating efficiently toward the peak regions.
Figure 2.19: Timelapse image series showing aligned cells pulling on local unaligned clusters ($\lambda = 1400 \mu m$, $A = 350 \mu m$). Cells aggregate into clusters before the pulling force from these aligned cells is sufficient to cause them to migrate toward the peak.

From the results of this study, we offer an explanation for a commonly observed phenomenon in cell culture, i.e. the meniscus phenomenon. This is an effect seen in three-dimensional gel cultures of epithelial cells, in which the cells form a circular ring of tubular structures near the perimeter of the plate, with acini on either side (Figure 2.20). This result can easily be explained with the stiffness gradient argument that we have developed in this study.
Figure 2.20: MCF10a and 3T3 cell co-culture in Matrigel™ on normal culture dishes. Pictured here is the meniscus region. Cells appear as acinar structures near the walls of the plate and at the center, but form duct-like structures parallel to the plate edges in the intermediate meniscus region. * Images taken by Michael Grigola and Ankita Tippur (Grigola and Hsia 2014)

Figure 2.21 shows the approximate stiffness gradient on the surface of the gel in the meniscus region.

Figure 2.21: Stiffness and stiffness gradient profiles of cells on meniscus region surface, with the left side (zero) representing the outer edge of the dish.
Because of the concave shape of the surface, the stiffness is highest near the edges of the plate where the gel thickness goes to zero. Moving away from the edge, the stiffness decreases, stabilizes, and then increases moving toward the inner boundary of the meniscus region. Finally, after reaching this inner edge, the stiffness becomes constant. Stiffness increases as you move to the center and eventually stabilizes at a higher, roughly constant value once the thickness of the gel becomes constant. Cells that are initially plated in the meniscus region, therefore, experience a stiffness gradient that favors migration either toward the edge or toward the center of the dish, according to the argument we have developed. Cells that migrate toward the center of the plate will stop when they reach the flat surface, where the stiffness becomes uniform and there is no longer a driving force behind migration. The result is a dense ring of cells that accumulates at the inner edge of the meniscus region. Once these cells are within close proximity, they begin to form tubular duct-like structures. The stiffness gradient argument provides a simple, yet elegant explanation for this common observation.

2.5 Conclusions

The method described above provides a simple technique for localizing cells to desired locations in soft three-dimensional extracellular matrix gels. The result shows promise for those wishing to control cell structure formation in three-dimensional gels for applications such as tissue engineering and regenerative medicine, cellular machines, and future work in the field of mechanobiology.
Further work is still needed to verify the linear relationship between stiffness gradient and cell migration velocity, and to better quantify collective effects. However, it is known that the cells migrate reliably toward regions of highest effective stiffness when gel thickness is below a certain level. The mechanisms behind this migration may be similar to those in two-dimensions; i.e. cells may prefer to migrate in a direction that provides the highest resistance to force. Future work should focus on determining the relationship of stiffness and stiffness gradient with the cell’s migration speed, as well as finding the underlying mechanisms responsible.
CHAPTER 3: PRESSURE EFFECT

3.1 Introduction

3.1.1 Background

Cells are exposed to a wide range of mechanical forces \textit{in vivo}, including unidirectional forces, such as tension and compression, twisting, shear, and hydrostatic pressure. Nature has been at work for millions of years, developing these forces and mechanisms in such a way that biologically appropriate survival responses can be induced within the cell. These responses enable cells and cellular systems to better survive and thrive through a variety of adaptations. We now know that externally and internally applied forces can modulate a number of cell behaviors, including, but not limited to, cell proliferation, differentiation, migration, adhesion, and even fate. The study of how and why these forces exist and what their effects may be is the basis of mechanobiology. In this section, we look specifically at the effects of a positive uniform hydrostatic pressure on cells, mimicking the elevated pressure found in many \textit{in vivo} tissues.

3.1.2 Applications of Cellular Forces \textit{in vitro}

In recent years, researchers have gained interest in the application of forces to cells
to study a variety of responses. Several unique tools and methods have been developed to cleverly probe the mechanotransduction of cells through application of various forces. Some researchers focus on collecting data from individual cells, requiring precise tools that can apply well-controlled forces, such as optical tweezers. On the other hand, some labs use larger bioreactors, or machines that apply a specified force to cells in bulk while maintaining biological conditions. The second approach yields the mean response of these cells, while foregoing some information about the individual cell response. Despite losing information about the specific responses of each individual cell, this method represents a fast, inexpensive, and effective tool to collect data about cellular responses, while closely replicating conditions found in vivo. In particular, pathological conditions within tissues most commonly arise from changes in bulk forces, rather than individual cell anomalies, and therefore this method can provide an excellent in vitro disease model.

To date, research around cell mechanics has largely focused on changes induced by simple forces such as shear, tension, or compression. These forces are often easy to replicate through the use of bioreactors. Examples include fluid flow for shear forces and mechanically pressing cells between two membranes for compression. These systems can become rather sophisticated as additional layers of complexity are added to better mimic the in vivo environment. For example, cells that line the passageways in the body may be subjected to shear forces that are non-continuous. The cells that line blood vessels, called endothelial cells, experience a cyclic shear
force that ebbs and flows as blood is pumped by the heart. Applying similar conditions in vitro may help us gain better insights into how these conditions impact cell behavior, and how variations in typical behaviors elicit different and/or pathological responses.

3.1.3 Relevance of Pressure in the Body

Hydrostatic pressure is another type of force that is biologically relevant for cells and tissues. Applying a positive pressure is one of the simplest methods for applying compression force to cells in vitro. The force is applied uniformly to the cell, rather than to a single surface as it is in some compression bioreactors. Although uniaxial compression may be relevant in many tissues, such as load-bearing bone, pressure is abundant in many tissues as well, and is often related to changes in fluid volume, such as inside the womb during pregnancy. Thus, studying pressure effects may provide important insights into the development of normal and pathological states of cells in vivo.

Positive pressure is frequently observed in the body whenever there are changes in a local fluid composition or volume in or around the cell. For instance, when an injury occurs, the body’s immune system response leads to inflammation. Cells and fluids are concentrated at the site of the injury, resulting in swelling and increased pressure at the wound. The effect of this abnormally high pressure can become very
serious or even fatal, depending on the location and duration. When external perturbations occur to the head, such as in traumatic brain injuries or after surgery, swelling and inflammation can cause fluids to build up inside the head. The volume is constrained by the rigidity of the skull, and thus compressive intracranial pressure can result. Left untreated, this pressure can end up damaging or even killing brain cells.

Understanding more about pressure-related pathologies, such as the intracranial pressure discussed above, is an important prerequisite for enforcing the proper precautions and treatments for these maladies. Information about the exact pressure levels and mechanisms leading to cell death, for example, can help medical practitioners make more educated decisions that can lead to improved outcomes for patients. Furthermore, greater knowledge of these systems may help prevent elevated pressure in the first place, reducing the overall risk to the patient.

3.1.4 Elevated Pressure and Cancer

Cancer is an important example of a pathological condition related to pressure. Several clinical studies have shown that tumors contain an elevated interstitial pressure relative to their normal tissue counterparts, and that malignant tumors contain much higher pressure than benign tumors (Nathanson and Nelson 1994). The volume of a tumor typically contains only about 50% cancer cells, while the rest
of the tumor is made up of blood vessels and interstitium, a dense form of extracellular matrix (Jain 1994). Leaky, poorly distributed vessels, inadequate lymphatic drainage, and compression from the rigid interstitium result in elevated interstitial fluid pressure (IFP) which inhibits blood flow and presents a major challenge for delivering drugs to these tumors (Jain and Stylianopoulos 2010).

Furthermore, as these findings predict, there is also a direct correlation between tumor size and the internal fluid pressure in the center of the tumor (Boucher et al. 1995; Znati et al. 1996; Milosevic et al. 2001). In Nathanson and Nelson’s study, the mean interstitial fluid pressure was found to be 29 ± 3 mm Hg (0.56 ± 0.058 psi) in invasive breast tumors, while only 3.6 ± 0.8 mm Hg (0.070 ± 0.015 psi) in benign breast tumors and -0.3 ± 0.1 mm Hg (-0.0058 ± 0.0019 psi) in normal breast parenchymal tissue (Nathanson and Nelson 1994). Similarly, significant IFP differences have been found in other types of tumors, such as osteosarcoma (Ubo et al. 2005) and melanoma (Rofstad and Halsør 2002). This close correlation makes it possible to use interstitial pressure measurements as an early indicator of cancer progression and treatment efficacy (Ferretti et al. 2009).

3.1.5 Problems with Interstitial Fluid Pressure

Aside from providing a marker for tumor size and metastatic potential, elevated pressure also presents a problem in the treatment of cancer. Elevated interstitial
pressure makes drug delivery to tumors difficult, as blood flow to the area is extremely slow. What little volume of a systemically injected drug does reach the tumor does not accumulate at high enough dosages to be effective. As a result, progress toward being able to “cure cancer” has proved disappointing, despite the large number of drugs that have been proven effective at killing cancer cells through *in vitro* studies. Thus, much of cancer research in recent decades has focused on the ability to target drug delivery or release and on alternative methods of targeted treatment.

Because cancer cells experience constant elevated pressure, it is important to be able to simulate these conditions in a lab. Aside from the challenges of drug delivery, it is likely that the altered state cells experience when they are under pressure could influence other aspects of drug uptake and efficacy. Thus, any lab studying the treatment or behavior of cancer cells should use pressure to properly emulate physiological conditions. Many such labs have purchased or developed systems to apply pressure by compression between two membranes. While this is appropriate for cells that experience uniaxial compression, such as bone, it is not an adequate representation of *in vitro* cancer conditions related to the interstitial fluid pressure, which is not unidirectional.

This section of the thesis will briefly cover the design of a simple and inexpensive hydrostatic pressure chamber, developed in the Hsia lab by former student Casey
Dyck. It will then discuss the use of this device in studying cancer cell response to pressure. The design of this pressure chamber can be replicated inexpensively and with relatively small amount of effort from the drawings provided in Dyck and Hsia 2012 by labs wishing to apply pressure to their cells.

3.2 Pressure Chamber Design and Modifications

The pressure chambers used in these experiments were designed by Casey Dyck and advised by Dr. Hsia in 2012 (Dyck and Hsia 2012). There are two chambers capable of exerting pressure: one chamber that sits inside a commercially available incubator and enables long-term imaging of several samples at a time (PIC-1), and a second chamber which stands alone and has a transparent top and bottom, allowing the long-term live imaging of cells under pressure (PIC-2). Both chambers are able to maintain the necessary conditions for cell culture, including 5% CO₂, high humidity, and 37°C temperature. Pressure can be applied in the range of 0-400 kPa (0-58 psi).

3.2.1 PIC-1

PIC-1 draws air from the inside of the incubation chamber, so there is no need to warm, humidify, or add CO₂ to the air. There is an external pump that pumps air
into a water capture chamber en route to the pressurized incubation chamber in order to prevent water from clogging the regulators. For additional information, drawings, and a part list for the original design, see Dyck and Hsia 2012. The pressure chamber as it was originally designed can be seen below in Figure 3.1.

![Pressure Chamber](image1.png)

Figure 3.1: Images of pressure chamber as it was originally designed (Dyck and Hsia 2012). The chamber sits inside the incubator and connects to a pump through a hole in the incubator wall.
Most cancer studies require the use of pressures in the range of 0-2 psi, since this is the range of physiological pressures observed inside the body. The PIC-1 system as it was originally designed did not have sufficient resolution to set pressures at this level, so the chamber had to be modified to allow us to study physiological tumor pressure. This was done through the addition of a back pressure regulator for the range of 0-3.5 psi (McMasterCarr #99045K11) attached to the lid of the chamber. An additional pressure gauge with better resolution at low pressures was also installed (McMasterCarr #4026K3, 0-5 psi), with a regulator in front to prevent damage to the gauge (43275K16, 0.5-3.5 psi). The final internal set-up can be seen in Figure 3.2.

Figure 3.2: Modified pressure chamber with resolution for studies at 0-2 psi. Modifications include the addition of a lower pressure gauge and regulator.
One issue we encountered with this set-up was evaporation of media in the samples due to the high flow rate of air through the chamber. At this high flow rate, approximately 0.5 mL of liquid evaporated from a round 35 mm dish over the course of 24 hours. Reducing the amount of evaporation requires slowing the airflow through the chamber by decreasing the output of the pump. Rather than investing in a second low-capacity pump, a transformer can be used to reduce the output of the pump by running it at a fraction of maximum power. By adjusting the knob on the transformer, it is possible to maintain a reasonable flow rate at any level of pressure up to approximately 40 psi. This set-up is shown in Figure 3.3.

Figure 3.3: Pump plugged into transformer to lower output. Transformer should be set to approximately 60% power.
To achieve pressure in the range of 1-2 psi, it is best to set the transformer to approximately 60% power and then adjust the inner black plastic regulator knob attached to the chamber lid to achieve finer pressure control.

3.2.2 PIC-2

PIC-2 is a free-standing pressure chamber with the capability for long-term imaging on a microscope. Since the chamber does not operate inside a commercial incubation chamber, it must be independently humidified, heated, and injected with CO₂. Therefore, a humidification chamber is used to humidify the air, while various heating elements throughout the incubation chamber body and the humidifier chamber are used to heat the samples and the air being circulated in. The CO₂ levels are maintained by using a premixed gas cylinder with 5% CO₂. There is a flow meter on the back end that regulates how quickly air flows out of the system. Thus, the pressure can be controlled simply by setting a low flow rate out of the system and pumping air from the cylinder through to the desired pressure level. For additional information, drawings, and a part list for the original design, please see Dyck and Hsia 2012. The pressure chamber as it was originally designed can be seen below in Figure 3.4.
5% CO₂ from the gas cylinder is heated and humidified prior to reaching the cell chamber (Dyck and Hsia 2012).

Since the pressure level in the chamber is controlled by the relative flow rate of the cylinder and the flow meter, it is already possible to achieve very low pressures (0-2 psi) with little adjustment to the system. However, the regulator on the CO₂ cylinder was unable to resolve these pressure levels, so an additional digital pressure gauge (McMasterCarr #2798K21) was added to the side of the chamber to enable better resolution at lower pressures. This can be seen in Figure 3.5.
Figure 3.5: Digital pressure gauge used to measure low pressure on timelapse chamber.

3.3 Materials and Methods

3.3.1 Cell Culture

All cells were cultured prior to experiments on tissue-culture treated plastic dishes. Media composition varies per cell type, as described below. Cells were grown to 90% confluence and then lifted from plates using TRYP LE, which contains enzymes to remove cell adhesions and allow transfer to a new plate or experiment. Cells used in experiments were kept between passage numbers 15-25. During all timelapse videos, cells were maintained in an environment-controlled cell chamber (37°C, 5% CO₂).

3.3.2 Cell Types
We study four types of breast epithelial cells in this section: MCF10A, MCF7, MDA-MB-231 and HME496 cells. MCF10A cells (ATCC® CRL-10317™) are a non-tumorigenic mammary gland epithelial cell line derived from a 36-year-old Caucasian female. These cells are commonly studied as a non-tumorigenic analogue to the MCF7 (ATCC® HTB-22) cell line, which is a mammary gland adenocarcinomic epithelial cell line derived from a 69-year-old Caucasian female. MDA-MB-231 cells are also tumorigenic mammary gland adenocarcinoma epithelial cells. Both MCF7 and MDA-MB-231 cells were derived from a metastatic cancer site, however MDA-MB-231 cells are considered the most invasive of the above three cell lines (Tse et al. 2011). The final cell type we discuss in this section is HME496 cells. These are primary cells that were generously donated by Dr. Susan Clare from the tissue bank at Indiana University School of Medicine.

MCF10A media composition is 50/50 Dulbecco’s Modified Eagle’s Medium/Ham’s F12 Nutrient Mixture (DMEM/F12) with the following supplements: 5% Horse Serum, 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 5 U/ml penicillin, 100 µg/ml streptomycin. MCF7 and MDA-MB-231 cells were both cultured with Dulbecco’s Modified Eagle’s Medium plus 10% Fetal Bovine Serum. HME496 cells were cultured using WIT-P Media and Serum (Stemgent 00-0045-500).

3.3.3 2D and 3D Culture Methods
The two methods used in these experiments were 2D and 3D-On-Top cultures. 2D culture simply involves plating the cells directly on top of a plastic or glass tissue-culture treated dish. The 3D-On-Top Culture Assay is a 3D culture method which allows better visibility of cells under a microscope by plating all cells on a single plane. The method was developed by Genee Lee and colleagues (Lee et al. 2007), and involves adding a thick layer of Matrigel™ on top of a surface, plating cells on top, and then exchanging the media for growth media + 2% Matrigel™ once the cells have attached. The resultant cultures can be described by Figure 3.6:

![Figure 3.6: Illustration of 2D, 3D embedded, and 3D on-top protocols for plating cells (Lee et al. 2007).](image)

3.3.4 Cell Number Assay

A cell number assay is a simple technique for counting cells in order to determine doubling rate and other parameters. Cells can be lifted from the dish using a variety of enzymes. For 2D experiments, cells are lifted with Trypsin or TRYP LE. For 3D, cold dispase can be used to dissolve the matrix and leave a single cell suspension. After this step, an excess of media is added to the cells to neutralize the enzyme, and the suspension is centrifuged at 200g for 5 minutes to form a pellet. Excess media is removed from the top of the conical tube, and fresh media is added to a known final
volume. Cells and media are mixed well by pipetting up and down several times.

Next, media is pipetted into a hemocytometer and a total of ten squares are counted. The total number of cells in the dish is calculated with the equation below:

\[
\text{Cells/mL} = \text{Cells/Square} \times 10,000 \quad \text{Eqn. 2}
\]

This procedure is repeated five times for each copy of the experiment.

3.3.5 Scratch Assay

The final technique used in these experiments is a scratch assay (Liang et al. 2007). Cells are grown to confluence in a 2D monolayer, and then the plate is scratched to remove cells from areas of the plate. The cell migration rate into the uncovered areas is monitored. Scratches are typically made using a pipette tip or specialized scratch assay comb (Millipore, Product #17-10191). This technique is demonstrated in Figure 3.7.

Figure 3.7: Scratch assay protocol. Left: Scratch assay comb that can be used to create uniform scratch wounds. Right: One scratch made on a confluent plate of MDA-MB-231 cells.
In these experiments, a plastic fork coated in PDMS was used to scratch the surface with evenly spaced marks. Images were then taken at periodic increments to measure migration into the uncovered regions over time.

3.3.6 Analysis of Cell Migration

Cells were processed with a MATLAB script developed by Daniel Perlitz. The processing steps include a series of filters, binarization, dilation, hole filling, and removing small objects. Next, a best fit line is made for both boundaries of the scratch. The distance between these lines is estimated by taking the average distance in the direction perpendicular to their mean slope. Combining the results for each scratch, and average of these distances was found for each time point and cell type, and a best fit line was made to find the average migration speed of cells in each plate.

3.4 Results

3.4.1 100 kPa Cell Proliferation

Since the resolution of the pressure chamber was initially low, early experiments were done at 100 kPa (14.5 psi). Cells were plated in 2D culture at 50,000 cells/plate and allowed to proliferate for 3 days, 6 days, or 9 days (n=3). After each
set of experiments, cells were counted using a hemocytometer. The data indicated that 14.5 psi slowed the growth of both MCF10a and MCF7 cells, and that MCF10a cells were proliferating more quickly than MCF7. This result is seen in Figure 3.8.

![Cell Number vs. Time](image)

Figure 3.8: Plot of cell number vs. time for MCF10a and MCF7 cells under atmospheric pressure and 100 kPa.

3.4.2 Cell Morphology in 3D at Various Pressures

Cell morphology in 3D culture is an important indicator of how cells respond to pressure. In addition, it is physiologically relevant to consider pressures more similar to native tumor pressure, in the range of 1 psi. Thus, cells were plated in the
3D On Top Assay described in Section 3.3.3. Cells were maintained at either atmospheric pressure or elevated pressure, and differences in cell morphology and percent coverage were measured after 4 days. This was done at both 1 psi and 14.5 psi, with control plates at each pressure. These results can be seen in Figure 3.9.

![Graph showing percent coverage after 4 days for MCF10a and MCF7 cells at 0 psi, 1 psi, and 14.5 psi.](image)

**Figure 3.9:** Average percent coverage of MCF10a and MCF7 cells after 4 days at 0 psi, 1 psi, and 14.5 psi.

Comparison images of MCF10a cells at both 0 psi and 1 psi after 4 days under the conditions described above can be found in Figure 3.10, below.
Figure 3.10: Morphology of MCF10a cells on Day 4 of 0 psi (right) and 1 psi (left). The two images do not show any significant differences in morphology or coverage.

Primary HME496 cells were also subjected to pressure, and images of these can be seen below in Figure 3.11.

Figure 3.11: HME496 primary breast epithelial cells under 0 psi and 1 psi. There are no noticeable differences between the morphology of the two types of samples.

3.4.3 Cell Migration Speed With and Without Pressure

A scratch assay was used to determine how pressure affects cell migration speed for
different cell types. In these experiments, MCF10a, MCF7, and MDA-MB-231 cells were scratched and then imaged at periodic increments. A sample timelapse image series for these scratches on MCF10a monolayers can be seen below in Figure 3.12.

![Timelapse images for scratch assay](image)

Figure 3.12: Scratch assay timelapse image series for cells under 0 psi and 1 psi. From these images, it appears that wound healing is more rapid at 0 psi than at 1 psi.

The MATLAB program developed for the analysis of the scratch assay results produces images with a best fit line at each edge, as seen in Figure 3.13.
The average perpendicular distance between the two lines is then computed, and the mean velocity of each type of cell under both pressure and control conditions can be found. The average velocities are given in Figure 3.14, below.

![Figure 3.13: Best fit lines for cell edges from scratch assay protocol.](image)

![Figure 3.14: Average migration velocity computed for each cell type and pressure level.](image)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>0 psi</th>
<th>1 psi</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10a</td>
<td>25.8 ± 2.8</td>
<td>16.4 ± 2.3</td>
</tr>
<tr>
<td>MCF7</td>
<td>15.3 ± 2.1</td>
<td>11.9 ± 1.5</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>34.8 ± 2.2</td>
<td>21.1 ± 2.6</td>
</tr>
</tbody>
</table>
3.5 Discussion

The scratch assay and proliferation results above demonstrate the differences between the three cell types and their responses to pressure. Although MCF7 and MDA-MB-231 cells are both cancer cell lines, the MDA-MB-231 cells are clearly much more invasive than the MCF7 cells, based on the scratch assay results. Furthermore, the data illustrates the effect of applying pressure to the cells. Although proliferation was not affected by 1 psi of pressure in MCF10a and MCF7 cells (Figure 3.9), all cell types experienced reduced migration rate under a pressure level of 1 psi. In MCF10a and MDA-MB-231 cells, this loss of migration was much more significant. Migration rate decreased by 39% in MCF10a cells and 41% in MDA-MB-231 cells.

Since pressure is actually highest in tumors in vivo, this result is somewhat counterintuitive. One might expect that pressure would create conditions favorable to new growth and migration of cancer cells, leading to a positive feedback loop; however, this is not the case. Although the pressure did not affect proliferation, the migration potential of cancer cells, and thus their ability to invade, is significantly reduced under pressure. Nevertheless, this result is supported by other cancer researchers, such as Mina Bissell and colleagues, who found that cancer cells can be forced to revert to their normal phenotype under positive pressure conditions (Venugopalan et al. 2012). Thus, it is not surprising to find that pressure could
actually reduce the invasiveness of cancer cells, driving them toward a more “normal” phenotype. This information is potentially useful in clinical settings, where treatment options for cancer patients could be expanded to include methods of physically compressing tumors.

3.6 Conclusions

Cell migration in response to a “wound” is a process which is indicative of migratory behaviors in cancer cells. In this study, the response of both normal and cancer cells to pressure is a reduction in migration with no change in proliferation. These results are supported by previous studies which suggest a role of pressure in regulating cancer progression. However, future work should focus on further exploring this effect with different pressure levels to better understand the limitations of this conclusion. Nevertheless, this study demonstrates the ability of the simply designed pressure chamber described by Casey and Hsia 2012 to effect changes in cell behavior which support other researchers’ claims. The simplicity and effectiveness of this chamber makes it an essential component of any cancer research lab.
CHAPTER 4: CONCLUSION

4.1 Summary

Cell response to environmental cues can come in a variety of forms. Small perturbations in the native environment in which cells exist can lead to differences in survival, cell type, morphology, proliferation, and migration, influencing in vivo processes and disease states. In vivo, cell migration characteristics can affect the progression of cancer, wound healing abilities, and many other normal and pathological processes. In this thesis, we show that migration can be influenced by both stiffness gradient and pressure. Future applications may use this information to help develop tools or techniques to reduce damage caused by cancer and other diseases, as well as improve the wound healing function in autoimmune deficient patients.

4.2 Future Work

Future work should focus on improving the understanding of the limits of these cues, and how they can be used to benefit the future of medicine. This will require a more complete computational model, more data to better quantify the limits of the
conditions’ influence on cells, and translational work to help apply these findings to useful applications such as tissue engineering and medicine. Although it may be several years before these cues will be used in medicine, the understanding of this potential may lead to a number of immediate advances in the fields of tissue engineering and regenerative medicine, where researchers are making strides to restore function of vital organs and tissues in deficient patients.
REFERENCES


